AFLPs: genetic markers for paternity studies in newts (Triturus vulgaris)

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Short Notes

AFLPs: genetic markers for paternity studies in newts (*Triturus vulgaris*)

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DNA-based genetic markers can reveal paternity whenever the direct assignment of fathers to offspring is precluded by multiple matings and internal fertilisation (e.g. Hughes, 1998). Microsatellites are the current marker of choice in many behavioural studies, and have revealed important insights into genetic mating systems of European amphibians (e.g., Garner & Schmidt, 2003). However, the number of amphibian species for which the time-consuming designing of locus-specific microsatellite primers was successful is still limited (for a review see Jehle & Arntzen, 2002), and the cross-utilisation of existing markers to closely related taxa seems to have a particularly low success rate (as shown in ranid frogs: Primmer & Merilä, 2002). Allozymes can infer parentage without a species-specific protocol, but, due to their low degree of polymorphism, in mate choice experiments require the a priori screening of individuals (as conducted in Rafinski & Osikowsi, 2002). Dominant markers such as RAPDs successfully identified closely-related amphibian species and their hybrids (Zeisset & Beebee, 1998; Mikulicek & Pialek, 2003), but might be less suited to distinguish between closely related individuals with a putatively very high frequency of shared bands.

AFLPs (Amplified Fragment Length Polymorphisms) are dominant genetic markers based on a technique which has been available since about a decade (Vos et al., 1995; Mueller & LaReesa Wolfenbarger, 1999; Luccini, 2003). Briefly, DNA restriction fragments up to a few hundred base pairs in length are ligated to synthetic adaptors, and subsequently amplified using PCR primers complementary to the adaptors with a few additional, selective nucleotides at their ends. Due to the possibility of genome-wide scans of genetic diversity, the main application of AFLPs to free-living populations is presently seen in population genomics and genome mapping (Luikart et al., 2003). However, AFLPs also have the potential to infer parentage and individual relatedness. In the present paper, we document their use to identify paternity in the internally fertilising smooth newt (*Triturus vulgaris*), a key species for studies on mate choice and sperm competition (Halliday, 1998, and references therein).

The data presented in this paper are based on nine female and 19 male *T. vulgaris*, field-collected in 2003 near Vienna (Austria). Experimental matings took place in aquaria ($50 \times 30 \times 30$ cm). Females sequentially picked up spermatophores from between two and four males, and wrapped their eggs into anchored plastic strips provided (detailed data will be reported

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elsewhere). We determined paternity of 21-89 offspring per female.

DNA was extracted from single toes of adults (removed after experimentation), or sacrificed embryos stored in absolute ethanol, using a standard phenol-chloroform procedure (Sambrook & Russell, 2001) followed by storage at −20°C. Approximately 100 ng genomic DNA was digested with 1 unit each *Eco*R1 and *Taq*1, in a total reaction volume of 20 μl containing 2 μl 10× TA buffer (100 mM Tris-Ac pH 7.9, 100 mM MgAc, 500 mM Kac, 10 mM DTT), and 5 μg BSA (Bovine Serum Albumin), incubated at 37°C for 3 hours, followed by 65°C for 10 minutes. A 5 μl reaction mixture containing 0.5 units of T4 DNA Ligase, 1 μl 5× ligation buffer and 50 μM double-stranded adaptors of each *Eco*R1 (forward 5′-CTCGTAGACTGCGTACC-3′, reverse 5′-AATTGGTACGCAGTCTAC-3′) and *Taq*1 (forward 5′-GACGATGAGTCCTGAC, reverse 5′-CGGTCAGGACTCAT-3′) was immediately added to the digested DNA samples. Ligations of adaptors to restriction sites took place at 16°C overnight. Preselective PCR amplification was conducted in 10 μl reaction volumes, containing 2 μL of diluted (4:1) template DNA, 0.5 units *Taq* DNA polymerase (Thermoprime^{Plus}, Advanced Biotechnologies) in the manufacturer's buffer (final concentrations 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween), 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 5 μM *Eco*R1 and *Taq*1 primers, with sequences complementary to the adaptors followed by two selective nucleotides (Table 1). The reaction profile used was 120 s at 94°C, then 20 cycles of 94°C (30 s), 56°C (30 s) and 72°C (60 s) followed by 10 minutes incubation at 72°C. The selective PCR was performed in a 10 μl reaction volume containing 2.5 μl diluted (1:10) preselective PCR products, 2.5 mM $MgCl_2$, 0.5 units *Taq* DNA polymerase in the manufacturer's buffer (see above), and 5 μM selective *Eco*R1 (6-Fam fluorescently labelled) and *Taq*1 primers carrying four selective nucleotides each (Table 1), using the following touchdown thermal profile: 120 s at 94°C, followed by 13 cycles of 94°C (30 s), 65-56°C (30 s, decreasing by 0.7°C per cycle), 72°C (60 s), and 10 minutes at 72°C. Fragments were separated and visualised using an ABI 3730 capillary sequencer and scored using the software GeneMapper^{TM} Version 3.5 (ABI). The reliability of the obtained AFLP fingerprints was confirmed by genotyping all potential parents several times. Unambiguous paternity was assigned by alleles unique for putative fathers and absent in the maternal fingerprint.

Table 1. Primer sequences used in AFLP-based paternity analysis in *Triturus vulgaris*. Randomly selected bases priming the genomic DNA are in bold. In our study population, preamplification primers T01P2 and T02P2 in combination with selective amplificaton primers T101, T105, T106, T205 or T206, respectively, produced the most informative fingerprints.

Preamplification	
EcoR1PreP2	GACTGCGTACCAATTC**TC**
T01P2	GATGAGTCCTGACCGAA**C**
T02P2	GATGAGTCCTGACCGA**CA**
Selective amplification	
EcoR1P4	6-fam-GACTGCGTACCAATTC**TCTA**
T101	GATGAGTCCTGACCGAA**CGA**
T105	GATGAGTCCTGACCGAA**CAA**
T106	GATGAGTCCTGACCGAA**CAC**
T204	GATGAGTCCTGACCGA**CAAT**
T205	GATGAGTCCTGACCGA**CAAA**

Sixteen primer combinations were initially tested, each yielding up to 140 fragments between 50 and 585 bp in length. The dominant nature of AFLPs precludes a distinction between homo- and heterozygotes, and several loci are necessary for unambiguous paternity assignment. Five combinations (T101, T105, T106, T204, T205, Table 1) provided the most informative data, and displayed between two and seven polymorphic loci per family each (see Figure 1 for an example). One primer combination was usually sufficient to resolve paternity in a specific family, but several combination were required across all families (detailed paternity data will be presented elsewhere).

Despite AFLPs being of wide use in other organisms, only a handful of studies have applied them to amphibians (Voss et al., 2001; Riberon

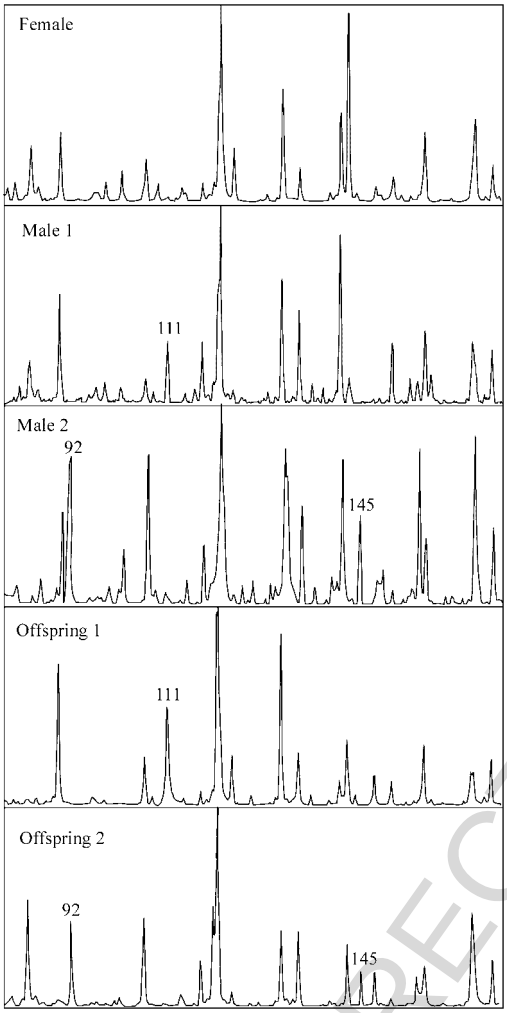

Figure 1. Example of an AFLP fingerprint (Primer combination T02P2 and T204, displaying dominant loci between 85 and 170 bp) to identify paternity in *Triturus vulgaris*. The size of diagnostic bands of two candidate fathers (absent in the known mother) are given in base pairs. Offspring 1 and 2 are sired by males 1 and 2, respectively.

et al., 2003; Curtis & Taylor, 2004; Bonin et al., 2004). To our knowledge we, for the first time, demonstrate their usefulness in amphibian parentage studies. Initial tests showed that amplifications with one and three selective nucleotides, the most commonly used AFLP protocol, yielded a large number of spurious bands and fingerprints which were often not interpretable (data not shown). The genome of urodeles is particularly large, and genome size is generally negatively correlated with PCR amplification success rates in microsatellites (Garner, 2002). We assume that the addition of a further selective nucleotide at both the preselective and selective amplification stage increased the reliability of priming in connection with the particluarly large genome of our study species. Related to this, no interpretable genotypes were obtained when slightly degraded DNA was used, and care should be taken that DNA is adequately stored. As only a small number of known candidate males was involved, paternity was assigned without probability-based statistical methods. In studies involving unobserved inseminations (such as in the field), however, more and potentially unsampled candidate fathers are usually involved, and/or the maternal genotype might be missing. In such cases, data across a significant number of primer combinations require to be incorporated into statistical frameworks particularly developed for dominant markers (Hardy, 2003; Hill & Weir, 2004; Wang, 2004), and the consequences of errors rates and/or rates of missing data would have to be carefully monitored (see also Bonin et al., 2004).

In conclusion, when applied with caution, AFLPs should provide a useful method for paternity studies in newts and other amphibian species for which polymorphic markers such as microsatellites are unavailable.

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